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TITLE: Modeling Marrow Failure and MDS for Novel Therapeutics

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14. ABSTRACT
Clonal evolution is a potentially life threatening long-term complication of inherited and acquired bone marrow failure (BMF) disorders. Cytogenetic clonal abnormalities develop in approximately 10% of patients with acquired aplastic anemia. The risk of early progression to myelodysplastic syndrome (MDS) and leukemia is also markedly elevated in patients with inherited marrow failure syndromes compared to age-matched controls. Prognosis of different clonal abnormalities is variable. Somatically acquired clonal deletions of one copy of chromosome 7 (monosomy 7) or part of the long arm of chromosome 7 (del7q) are generally associated with a poor prognosis. Deletions involving chromosome 7 are frequently associated with therapy-related MDS, hypocellular MDS, MDS arising in children, and in MDS arising in patients with marrow failure. The goal of this project is to model monosomy 7 arising in marrow failure by utilizing induced pluripotent stem cells (iPSC) derived from patients with the inherited marrow failure/leukemia predisposition syndrome Shwachman-Diamond syndrome (SDS) into which a deletion of the MDS-associated region of 7q has been genomically engineered. We will perform functional genomic screens to identify genes and molecular pathways with synthetic lethality for the del7q clone. These studies will provide a platform for the development of new strategies to treat monosomy 7 clonal disease arising from marrow failure. In light of recent reports describing methodologies to expand iPSC-derived CD34+ cell populations, during the project period, we investigated the possibility of conducting the screen using hematopoietic CD34+ cells. succeeded in expanding CD34+ cells from the SDS iPSC; however, the iPSC carrying the deletion of chromosome 7q failed to expand in sufficient quantities required for screening. Although the effort spent working out the CD34+ expansion required some additional time, we are still on track to complete the aims. We are proceeding with the screen using undifferentiated SDS iPSC+/-del7q. We have validated the feasibility of expanding the del7q clones for high throughput genomic screening and are in the process of conducting the screen using Cas9 with a guide RNA library as outlined in the proposal.

15. SUBJECT TERMS None listed

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1. Introduction

Clonal evolution is a potentially life threatening long-term complication of inherited and acquired bone marrow failure (BMF) disorders. Cytogenetic clonal abnormalities develop in approximately 10% of patients with acquired aplastic anemia. The risk of early progression to myelodysplastic syndrome (MDS) and leukemia is also markedly elevated in patients with inherited marrow failure syndromes compared to age-matched controls. Prognosis of different clonal abnormalities is variable. Somatically acquired clonal deletions of one copy of chromosome 7 (monosomy 7) or part of the long arm of chromosome 7 (del7q) are generally associated with a poor prognosis. Deletions involving chromosome 7 are frequently associated with therapy-related MDS, hypocellular MDS, MDS arising in children, and in MDS arising in patients with marrow failure. The goal of this project is to model monosomy 7 arising in marrow failure by utilizing induced pluripotent stem cells (iPSC) derived from patients with the inherited marrow failure/leukemia predisposition syndrome Shwachman-Diamond syndrome (SDS) into which a deletion of the MDS-associated region of 7q has been genomically engineered. We will identify genes and molecular pathways with synthetic lethality for the del7q clone. These studies will provide a platform for the development of new strategies to treat or prevent monosomy 7 clonal disease arising from marrow failure.

2. Keywords

Bone marrow failure, clonal evolution, induced pluripotent stem cells, genomic engineering

3. Accomplishments

What were the major goals of the project?

The goal of this study was to understand the biology of del7q MDS in the context of bone marrow failure, using Shwachman Diamond syndrome as the disease model, and to identify targeted vulnerabilities to treat or prevent MDS.

What was accomplished under these goals?

In preparation for a CRISPR screening assay, we successfully introduced a stably expressed CRISPR cDNA into the SDS +/- del7q induced pluripotent stem cells (iPSC) which was well tolerated (Figure 1A and 1B). CRISPR activity was demonstrated using a GFP target (Figure 1C). The cells maintained a stable karyotype and did not exhibit a change in their growth patterns.

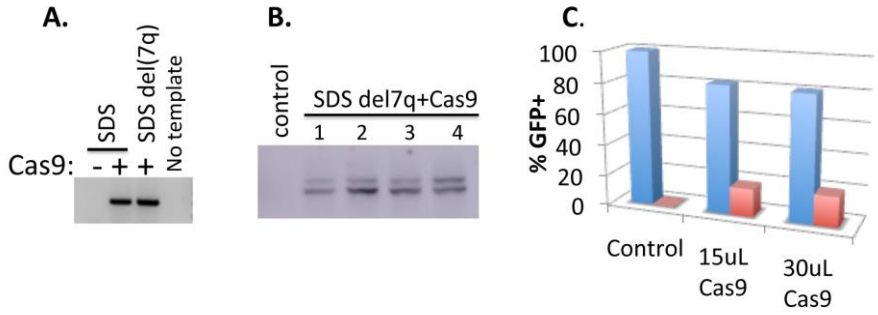


Figure 1. Stable CRISPR expression and activity in SDS +/-del(7q) iPSC. A. PCR for Cas9 expression in iPSC (SDS or SDS del(7q)) transduced with Cas9 lentivirus as indicated. B. Western blot for Cas9 protein expression in iPSC . Control = iPSC without Cas9 transduction. C. CRISPR activity measured by GFP target expression with or without introduction of anti-GFP guide RNA.

In preparation for the screen, we pursued a strategy to expand the iPS-derived CD34+ hematopoietic stem/progenitor population with the intent to conduct this screen in a hematopoietic cell type rather than in the pluripotent state. This strategy developed by the laboratory of George Daley introduces a set of 5 cDNAs (ERG, HoxA9, RORA, SOX4, MYB) which are doxycycline-inducible (Doulatov et al, 2013, Cell Stem Cell 13(4):459-70). Unfortunately, the SDS iPSC-derived CD34+ cells exhibited limited expansion capacity which was even further reduced in the SDS del(7q) line (data not shown).

The additional challenge that arose was the propensity of the del7q clone to retain an additional copy of chromosome 7 to restore a diploid 7q state. This occurred both during the generation of additional del7q lines, resulting in a mixed population, as well as during propagation of the established del7q clone. For this reason, we repeatedly tested the del7q lines prior to experiments to ensure maintenance of the del7q karyotype. (Figure 2).

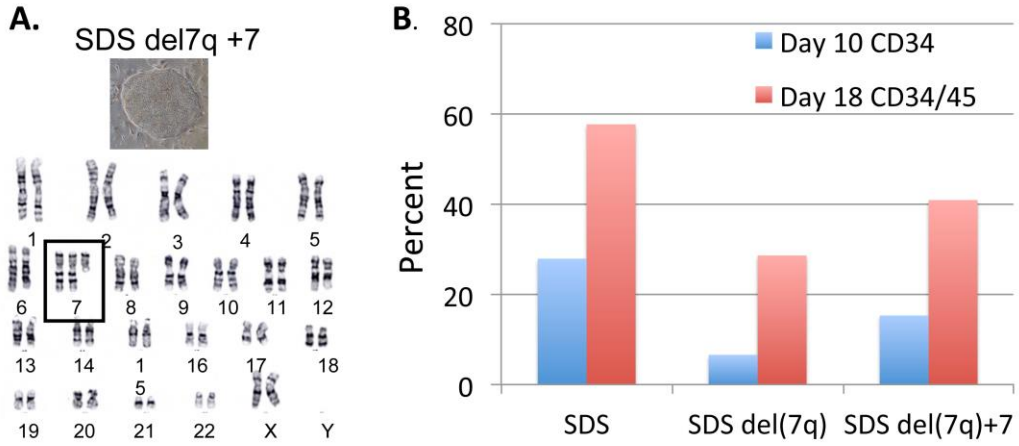


Figure 2. Spontaneous reversion of SDS del(7q) iPSC. A. Karyotype of spontaneous reversion of del(7q) clones. B. Restoration of 7q diploid status improves hematopoiesis. The indicated iPSCs were differentiated to CD34+ (day 10) and CD34+/45+ (day 18) hematopoietic cells and quantitated by flow cytometry.

Since somatic acquisition of p53 mutations is common in SDS, we queried the effect of p53 on hematopoiesis of SDS and found that p53 knockdown in primary SDS patient-derived bone marrow samples rescued bone marrow failure (Figure 3). We therefore knocked out p53 in the SDS iPSC +/-del7q and found that, in contrast to the improved function of CD34+ SDS cells, p53 knockout did not improve the production of CD34+ cells nor did this improve hematopoietic differentiation of the iPSC in these initial experiments (data not shown). We observed frequent karyotype abnormalities in SDS iPSC following p53 knockdown.

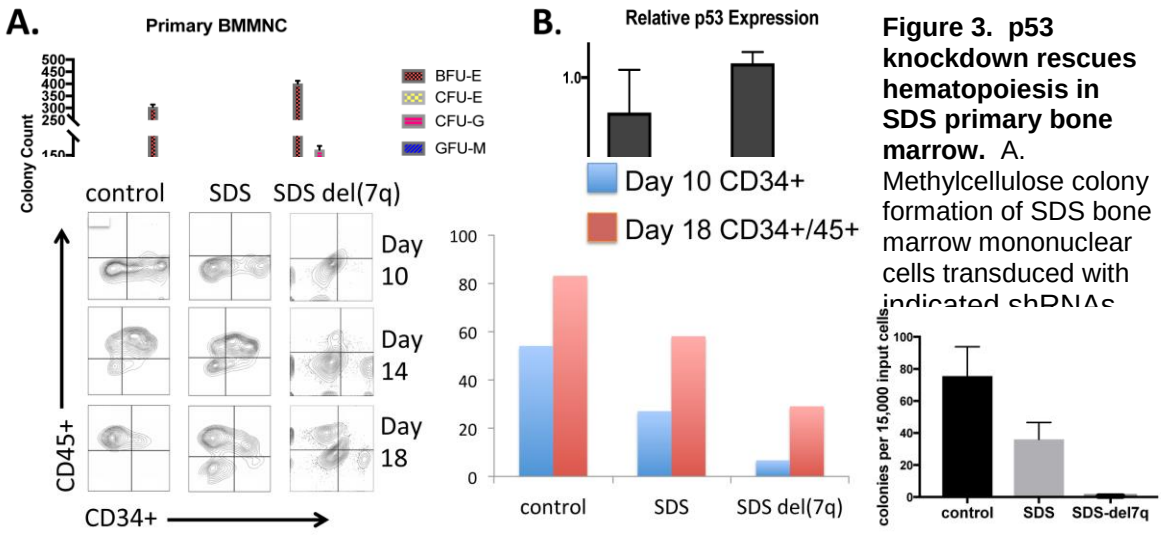


Figure 3. p53 knockdown rescues hematopoiesis in SDS primary bone marrow. A. Methylcellulose colony formation of SDS bone marrow mononuclear cells transduced with indicated shRNAs

We found that deletion of 7q failed to confer a relative growth advantage in either the pluripotent state or following differentiation to CD34+ cells (Figure 4). These data are consistent with recently emerging clinical data that monosomy7/del7q clones may be transient. Taken together, these data indicate that additional events, such as driver mutations, are required to progress to MDS and leukemia.

Figure 4. Deletion of 7q fails to improve hematopoiesis of SDS iPSC. Left: Flow cytometry of iPSC hematopoietic differentiation. Middle: quantitation of CD34+ (day 10) and CD34+/CD45+ (day 18) iPSC-derived hematopoietic cells. Right: Methylcellulose colony assay of iPSC-derived hematopoietic progenitor colonies

Given the genomic instability of the SDS del7q cells, the CRISPR screen was ultimately not successful; nonetheless, these studies identified a potential targeted vulnerability resulting from the differential state of the TGFβ pathway in the SDS +/-del7q lines. (Figure 5 and Figure 6)

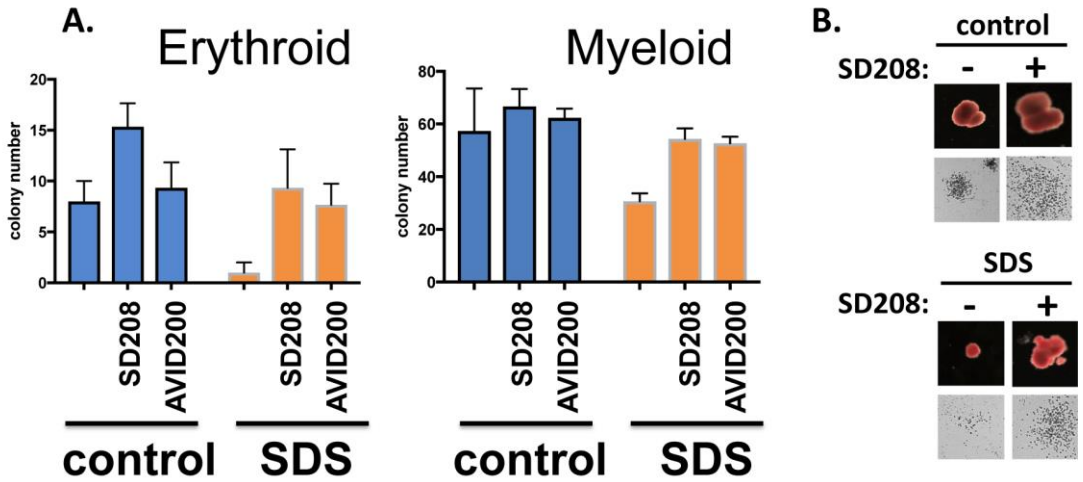


Figure 5. TGFβ inhibition rescues hematopoiesis of SDS bone marrow. A. Methylcellulose colonies of SDS bone marrow mononuclear cells treated with the TGFβ inhibitors SD208 or AVID200. B. TGFβ inhibition results in improved colony size.

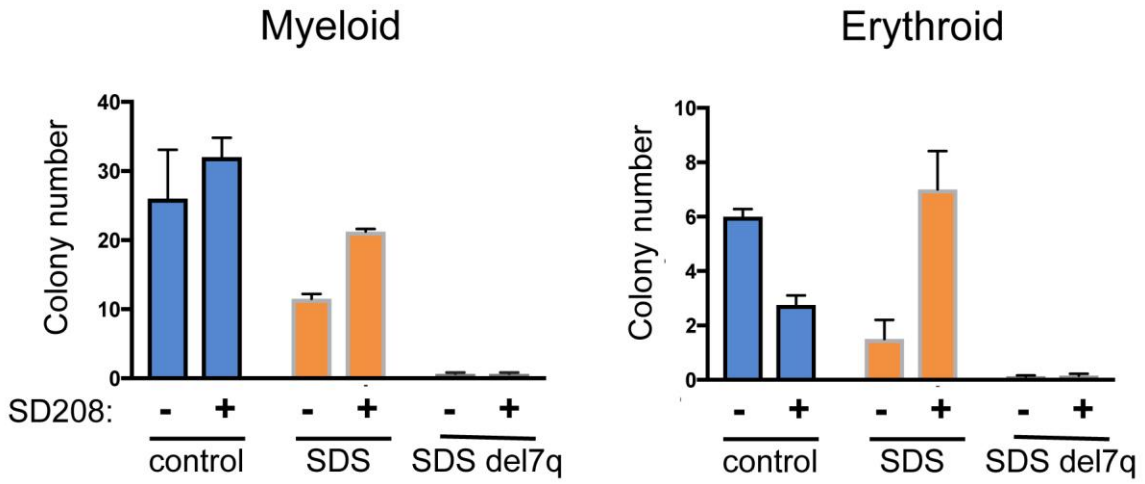
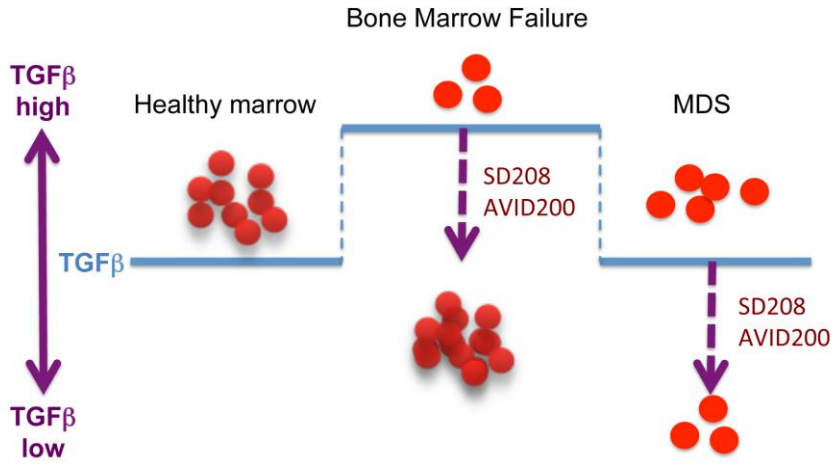


Figure 6. TGFβ inhibition rescues hematopoiesis of SDS but not of SDS del(7q) iPSC.

This observation was intriguing since TGFβ pathway is upregulated in MDS and TGFβ inhibitors are currently in clinical trials to treat MDS. These data suggest a model (Figure 7) whereby TGFβ pathway is activated in SDS resulting in suppression of hematopoiesis but is downregulated following deletion of 7q. Although TGFβ inhibition restores normal pathway activity in SDS to improve hematopoiesis, the TGFb pathway presents a targetable vulnerability in the del7q cells. A clinical trial of TGFβ inhibitors to treat SDS is currently being developed. In addition, as noted in our previous progress report, we are utilizing this iPS model to systematically introduce somatic mutations observed in SDS during progression to MDS and leukemia to understand the functional consequences of specific somatic mutations.

Figure 7. Model of targeted vulnerabilities in MDS secondary to bone marrow failure



What opportunities for training and professional development has the project provided?
This project provided training for Dr. Melisa Ruiz-Gutierrez, MD PhD, who is an under-represented minority female trainee, as well as for Dr. Ozge Bolukbasi, a postdoctoral fellow. A manuscript of these studies is currently in preparation.

How were the results disseminated to communities of interest?
To disseminate these results to communities of interest, these data were presented in several invited oral presentations given by me and by Dr. Ruiz-Gutierrez at meetings including the American Society of Hematology in Atlanta, GA in December 2017, European School of Hematology meeting in Estoril, Portugal in October 2017, the plenary session at the American Society of Pediatric Hematology Oncology in Pittsburgh, PA in May 2018, and the 12th Myeloid Biology meeting in Cincinnati OH.

What do you plan to do during the next reporting period to accomplish the goals?
This is the final report so no further plans will be outlined here. Future studies will introduce patient-derived somatic mutations to understand the biology of clonal evolution in bone marrow failure.

4. Impact
What was the impact on the development of the principal disciplines of the project?
Our studies demonstrate that deletion of 7q alone failed to confer a competitive growth advantage in the context of bone marrow failure and therefore additional somatic mutations are likely required for disease progression. These studies also identified the TGFβ pathway as a potential targetable pathway to treat bone marrow failure without promoting the outgrowth of del(7q) cells.

What was the impact on other disciplines? Not applicable
What was the impact on technology transfer? Not applicable
What was the impact on society beyond science and technology? These studies provide preclinical data to support the development of a clinical trial.

5. Changes/Problems
Changes in approach and reasons for change.
Extensive efforts eventually led us to conclude that the originally proposed screening approach would not be feasible with the del(7q) lines. Therefore, we refocused the experiments on differentially regulated pathways to successfully identify targetable vulnerabilities in the del(7q) line as describe above.

Actual or anticipated problems or delays and actions or plans to resolve them
As noted in section 3, the del(7q) lines were genomically unstable and also failed to expand in the CD34+ background; nonetheless, we were able to identify novel pathways by studying the biology of the SDS +/-del(7q) iPSC to improve bone marrow failure without promoting outgrowth of the del7q MDS cells.

Changes that had a significant impact on expenditures. None.
Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents. None.
Significant changes in use or care of human subjects. None.
Significant changes in use or care of vertebrate animals. None.
Significant changes in use of biohazards and/or select agents. None.

6. Products

Publications, conference papers, and presentations

This work was presented in an invited oral presentation at the European School of Haematology in October of 2017, an oral platform session at the annual meeting of the American Society of Hematology in December 2017, the plenary session of the American Society of Pediatric Hematology Oncology in May 2018, and in an invited oral presentation at the 12th International Workshop on Molecular Aspects of Myeloid Stem Cell Development and Leukemia in Cincinnati Ohio in May 2018. A manuscript of this work has been drafted and submission is planned in the coming month.

Websites or other Internet sites. None.
Technologies or techniques. None.
Inventions, patent applications, and/or licenses. None.
Other products. None.
Other products

The SDS+/-del7q induced pluripotent stem cells provide a useful cell model to study the biological effects of deletion of 7q. To my knowledge, this is the only isogenic cell line +/- del7q to model MDS in bone marrow failure.

7. Participants and other collaborating organizations

Name: Akiko Shimamura, M.D., PhD
Project Role: Principle Investigator
Researcher Identifier: ORCID 0000-0002-4683-9958
Nearest person month worked: 1.2 calendar months
Contribution to project: Dr. Shimamura designed the project, analyzed the data, interpreted the experiments and is drafting the manuscript.
Funding support: Supported by other research grants from NIH/NHLBI, Fanconi Anemia Research Foundation, Leukemia Lymphoma Society, and NIDDK.

Name: Marilyn Sanchez Bonilla, MS
Project Role: Research Scientist
Researcher Identifier: N/A
Nearest person month worked: 6.0 calendar months
Contribution to project: She engineered the del7q deletion in a second iPSC line derived from an SDS patient and assisted Dr. Ruiz-Gutierrez with the functional assays
Funding support: N/A

Name: Melisa Ruiz-Gutierrez, M.D., PhD
Project Role: Physician scientist postdoctoral fellow
Researcher Identifier: N/A
Nearest person month worked: no salary support was requested since Dr. Ruiz-Gutierrez was supported by a K12 training grant.
Contribution to project: Her background training spans molecular biology, biochemistry, cell biology, and induced pluripotent stem cell biology. She performed the hematopoietic differentiation and functional assays for this study and is drafting the manuscript.
Funding support: 4K12HL087164-10 (NIH)

Name: Ozge Vargel-Bolukbasi, PhD
Project Role: Research Fellow
Researcher Identifier: N/A

Nearest person month worked: calendar months
Contribution to project: She worked with Dr. Ruiz-Gutierrez on the preparation for the screen and functional assays.
Funding support: Leukemia Lymphoma Society

Name: Kaitlyn Ballotti, BS
Project Role: Research Assistant
Researcher Identifier: N/A
Nearest person month worked: calendar months
Contribution to project: Miss Ballotti maintained the iPSC cultures. These cells are labor-intensive to culture and required ongoing daily time-consuming work.
Funding support: N/A

Has there been a change in the active other support of the PI since the last reporting period? Yes

What other organizations were involved as partners?
No collaborating organizations to report.

8. Special reporting requirements
Not applicable

9. Appendices
Not applicable